Factors impacting unbound vancomycin concentrations in different patient populations

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Running title: unbound vancomycin in different patient populations

Keywords: Protein binding, vancomycin, unbound concentrations, LC-MS/MS, Therapeutic Drug Monitoring

Abbreviations:

MIC : Minimal Inhibitory Concentrations; TDM: Therapeutic Drug Monitoring; CKD-EPI : Chronic Kidney Disease Epidemiology collaboration equation; ICU: Intensive Care Unit; IgM: Immunoglobulin M; IgA: Immunoglobulin A; AAG: Alpha-1-Acid-Glycoprotein; PPB: Plasma Protein Binding; HPLC: High Performance Liquid Chromatography; LC-MS/MS: Liquid Chromatography-Tandem Mass Spectrometry; FPIA: Fluorescence Polarization Immunoassay; ME : Matrix Effect; PETINIA: Particle Enhanced Turbidimetric Inhibition Immunoassay; NSAID: Non-Steroidal Anti-Inflammatory Drugs; UV: Ultra Violet; LOQ: Limit of Quantification; IS: Internal Standard; SD: Standard Deviation; SE: Standard Error

Abstract

Introduction

The unbound drug hypothesis states that only unbound drug concentrations are active and available for clearance and highly variable results regarding unbound vancomycin fractions are reported in literature. We determined unbound vancomycin fractions in four different patient groups using a Liquid Chromatography Mass Spectrometry (LC-MS/MS) method and identified factors that modulate vancomycin binding. We further developed and validated a prediction model to estimate unbound vancomycin concentrations.

Materials and methods

Vancomycin (unbound and total) concentrations were measured in 90 patients from 4 different patient wards (hematology (n=33 samples); intensive care unit (ICU) (n=51); orthopedic (n=44) and pediatrics (6 months to 14 years, n=18)) using a validated LC-MS/MS method. Multiple linear mixed model analysis was performed to identify patient variables that were predictive for unbound vancomycin fractions and concentrations. Variables included in the model were age, patient ward, number of co-administered drugs with high protein binding, kidney function (estimated glomerular filtration rate (CDK-EPI formula)), alpha-1-acid-glycoprotein, albumin, total bilirubin, IgA, IgM, urea, and total vancomycin concentrations.

Results

In the pediatric cohort, median unbound vancomycin fraction was 81.3% (range: 61.9-95.9%), which was significantly higher ($p<0.01$) than the unbound fraction found in the three adult cohorts (hematology (60.6% (48.7-90.6%)), ICU (61.7% (47.0-87.6%)) and orthopedic (56.4% (45.9-78.0%)) patients). The strongest significant predictor for unbound vancomycin concentration was the total concentration, completed by albumin in the pediatric cohort, and albumin and IgA in the adult cohorts. Validation of our model was performed in 13 adult patients. A mean difference of 0.3 mg/L (95%CI: -

1.3 - 0.7 mg/L; R^2=0.99 (95%CI: 0.95-0.99)) between measured and calculated unbound vancomycin concentrations demonstrated the predictive performance of our model was favorable.

Conclusion

Unbound vancomycin fractions vary significantly between pediatric and adult patients. We developed a formula to estimate the unbound fraction derived from total vancomycin, albumin and IgA concentrations in adult patients.

1. Introduction

Vancomycin, a glycopeptide antibiotic, is widely used to treat infections caused by methicillin-resistant *Staphylococcus aureus* and other β-lactam resistant gram positive cocci.[1,2] The potential rise in minimum inhibitory concentrations (MICs) of vancomycin makes it increasingly important to adjust its dose in order to ensure adequate concentrations in blood and other infected areas as well as to avoid undue toxicity.[3,4] Generally, therapeutic drug monitoring (TDM) focuses on the total drug concentration in human plasma or serum, although, it is hypothesized that only the "free" or "unbound" fraction of the total concentration is responsible for antimicrobial activity, potential toxicity and available for clearance.[5-8]

Vancomycin is generally considered as a moderately protein bound antibiotic (30-60%), with albumin being an important binding protein.[7] A protein binding of 50% is generally considered to calculate unbound vancomycin concentrations. However, protein binding of vancomycin shows considerable variability across studies (ranging from almost 0 up to 90%), which could lead to different clinical responses even within the same total concentration.[9-14] Unbound drug concentrations can vary among patients (hematology, intensive care, pediatric, etc.) and underlying disorders (burn, myeloma, obese patients), possibly resulting in different responses to therapy or toxicity as only the unbound drug concentrations are considered pharmacologically active. Previous studies examining the correlation between unbound and total vancomycin concentrations did not distinguish between different patient populations.[9,12,14] One study investigated only intensive care patients.[10] Bertoin *et al* measured unbound and total concentrations in three patients groups (i.e. hematology, intensive care and orthopedic patients). [11] No differences in unbound concentrations between the three groups were demonstrated, but the number of patients studied in the different groups was rather low.[11] Moreover, none of the different studies investigated unbound and total concentrations in pediatric patients. Given the different behavior of drugs in this patient group, it is of specific interest to measure total and unbound concentrations in these patients.

100 In this study, we evaluated unbound vancomycin fractions in a larger cohort of different patient
101 populations and identified factors associated with unbound vancomycin concentrations using a
102 validated LC-MS/MS method for measurement.

Antimicrobial Agents and Chemotherapy

2. Materials and methods

2.1. Method validation

2.1.1. Vancomycin determination

Unbound vancomycin concentrations were determined using Centrifree Centrifugal Filter Devices (Millipore, Billerica, US: MWCO 30.000). Briefly, fresh lithium-heparin plasma (600 µL/sample) samples were incubated in a capped Centrifree Device during 30 minutes, after which the device was spun at 1912 *g* for 30 minutes at 37°C in a pre-conditioned Sigma 3-18K centrifuge (SciQuip, London, UK). The unbound vancomycin concentration is the concentration measured in the ultrafiltrate. Unbound as well as total vancomycin concentrations were determined using the chromatographic conditions of a recently described method (within run imprecision: 2.5-5.2%; total imprecision: 2.6-8.5%; limit of quantification: 0.3 mg/L).[15] We further validated this method for determining unbound vancomycin concentrations. The unbound vancomycin fraction (%) was calculated as ultrafiltrate concentration/total vancomycin concentration x 100%.[14]

2.1.2. Analytical validation of LC-MS/MS method for unbound vancomycin concentrations

Method imprecision was evaluated by analysis of two randomly selected left-over patient samples (mean unbound and total vancomycin concentrations: 3.0 and 12.2 mg/L; 4.3 and 18.6 mg/L, respectively) on 10 consecutive runs on 10 different days.

Accuracy was performed by measuring unbound vancomycin concentrations in two spiked ultrafiltrates (5 and 20 mg/L) in ten different runs. The percentage deviation from the theoretically added vancomycin concentration was calculated. An accuracy of <15% was accepted.[16]

Extraction recovery of the ultrafiltrate was evaluated by comparing the peak areas of vancomycin spiked in ultrafiltrate both before and after sample preparation (loss during extraction). The ultrafiltrate was spiked with three concentrations, i.e. 5, 20 and 40 mg/L. Matrix effect (ME) was

evaluated by comparing the peak areas of vancomycin spiked with 5 and 20 mg/L in pure solvent (water), with the peak areas of vancomycin spiked with 5 and 20 mg/L in ultra-filtrate of one blank plasma sample (healthy volunteer) and two different patient left-over plasmas. The sample ME was calculated with the equation ME% = B/A*100, where B refers to the peak area of vancomycin obtained in matrix and A to the peak area in solvent. Recovery of the UF membrane (adhesion of vancomycin at the membrane) was assessed by measuring in triplicate spiked ultrafiltrate at three different vancomycin concentrations (5, 15 and 30 mg/L) before and after filtration (second ultrafiltration).

Antimicrobial Agents and Chemotherapy

2.2. Correlation study

2.2.1. Patient population

A retrospective study was conducted at the University Hospitals Leuven on left-over samples from patients who received vancomycin for a suspected or proven gram-positive infection and required TDM (routine total vancomycin) between April and June 2014. Unbound and total vancomycin concentrations of patients admitted at the hematology, intensive care, orthopedic and pediatric (patients aged between 6 months and 14 years) wards were measured. Eight patients received continuous infusion, 82 patients were treated with intermittent infusion of vancomycin.

2.2.2. Data collection

The following baseline data were collected from the laboratory information system: age, gender, underlying condition, diagnosis upon admission and underlying condition. Treatment details consisted of vancomycin daily dose and route of administration, co-administered drugs at the day of sampling, with special focus on those with high plasma protein binding (PPB) (above 70%), such as vitamin K antagonists, aspirin, Non-Steroidal Anti-Inflammatory Drugs (NSAIDs), phenytoin and valproic acid.[17] To each different drug, a score of 1 was attributed. These summations were further included in the multivariate analyses.

Biochemical findings at the day of sampling included albumin, bilirubin (direct and total), creatinine, glomerular filtration rate as estimated by CKD-EPI, total protein, urea, IgA, IgM, alpha-1-acid-glycoprotein (AAG) and total plasma vancomycin concentration. These biochemical data were obtained from the laboratory information system from samples with identical sampling time as the sample for total routine vancomycin measurement. If one of the biochemical parameters was missing, an additional measurement was performed on left-over material (serum or lithium-heparin plasma) with the same sampling time as the sample for vancomycin determination. All described biochemical parameters can be measured on serum as well as on lithium-heparin plasma (evidenced by the

respective method insert sheets from the manufacturer). The study was approved by the Ethics Committee of University Hospitals Leuven. As the study was performed on left-overs, informed consent of each subject was hereby waived.

2.2.3. Methods

Left-over lithium-heparin plasma samples, sent to the clinical laboratory of the University Hospitals Leuven for clinically indicated total vancomycin measurements, of different patient groups were collected from the laboratory sample storage system (2-8°C) at the end of the working day. Samples were centrifuged during 10 minutes at 1920 *g* (20°C). Only recent samples (same day) were selected. Stability of vancomycin in plasma has been evidenced in these conditions and even for longer periods.[15] An aliquot was frozen for measurement of total vancomycin using LC-MS/MS (-20°C) and another part was processed directly to obtain the unbound vancomycin fraction. Therefore, 600 µL lithium-heparin plasma was centrifuged through a Centrifree UF device and stored at -20°C until analysis. Unbound and total vancomycin concentrations were measured by LC-MS/MS.[15] Albumin (bromocresol), bilirubin (direct and total) (diazonium, colorimetric), creatinine (enzymatic IDMS traceable), total protein (biurete) and urea (kinetic urease-glutamate dehydrogenase) concentrations were determined on a Cobas 8000 c702 analyzer (Roche Diagnostics, Mannheim, Germany) in clinical setting or on left-over lithium-heparin plasma or serum (left-over collected and processed as described for vancomycin). IgA, IgM and AAG analyses were performed on an IMAGE nephelometer (Beckmann Coulter, Brea, CA, USA).

2.2.4. Statistical analysis

Statistical analysis was performed using SPSS 22.0 for Windows (SPSS Inc 2011, Chicago, IL). A *p*-value <0.05 was considered significant. Bland-Altman analysis, Passing & Bablok regression and Spearman correlation coefficients were determined using Medcalc version 11.6.1.0. (Medcalc, Ostend, Belgium). Data were described with means ± standard deviations (SD) or medians and IQR according to the data distribution. Univariate correlations were investigated using scatterplots combined with Spearman's rank correlation coefficients. Multivariate analysis was conducted using linear mixed modeling with random intercept. Spearman's rank correlation coefficients were used instead of Pearson correlation coefficients because no assumptions of linearity were made in advance. However,

Antimicrobial Agents and Chemotherapy

because linear mixed models were used to create the prediction formula, Pearson correlation coefficients could also be used in this analysis. Differences between different patients groups were testing using a Mann-Whitney U test.

202 **2.3. Model validation**

203

204 The performance of the prediction model was assessed to determine the validity of the unbound
205 vancomycin estimate (on left-over samples). The study cohort for validation of the prediction tool
206 consisted of another 13 hospitalized adult patients of three different adult patient wards with a
207 clinically indicated vancomycin sample. Mean differences were calculated as the absolute difference
208 between calculated and measured unbound vancomycin concentrations, divided by the measured
209 concentration.

3. Results

3.1. Method validation

The resulted mean unbound vancomycin CVs were 3.0 and 4.4%, respectively. Accuracy ranged from 99.9 to 107.5 % for the different concentrations. The average extraction recovery was 98.9% (1.4% CV) for unbound vancomycin. ME ranged from 61.8 to 67.7% and 49.0 to 71.4% for the 5 mg/L and 20 mg/L spiked ultrafiltrates, respectively. When the response ratios (RR, i.e. area vancomycin/area internal standard (IS)) were calculated, ME ranged from 94.9 to 106.2% and 93.2 to 103.7% (5 and 20 mg/L, respectively) as compared to the RR in pure solvent, indicating acceptable compensation of the IS. Non-specific adsorption of vancomycin to the Centrifree UF membrane was minimal and ranged from -2.4 to 0.9%, depending on the concentration. The initial unbound vancomycin fraction was 65.8 and 60.7% before the samples were frozen, 64.2 and 58.3% after three days, and 65.2 and 59.4% after one week thawing (mean total vancomycin concentrations 7.4; 16.5 and 22.8 mg/L). These results indicate that the freeze-thaw process does not substantially alter the vancomycin PPB.

3.2. Correlation study

Table 1 displays baseline demographic, clinical and biochemical data of patients included in the study. In the total population, median total vancomycin concentration was 13.5 mg/L (range: 0.7-53.2 mg/L), median unbound vancomycin concentration was 8.5 mg/L (0.6-34.2 mg/L). The median unbound vancomycin fraction was found to be 81.3% (range: 61.9-95.9%) in the pediatric cohort, which was significantly higher compared to the fractions obtained in the three adult cohorts (hematology (60.6% (48.7-90.6%)), ICU (61.7% (47.0-87.6%) and orthopedic patients (56.4% (45.9-78.0%)) (Figure 1A) (Mann Whitney U test). Except for the ICU population, no significant differences in total vancomycin concentrations between the different populations were found (Mann Whitney U test) (Figure 1B).

In the entire population, unbound vancomycin concentrations correlated very strongly with total vancomycin concentrations (R = 0.96; p <0.01). Besides, a moderate correlation was found with urea (R = 0.42; p<0.01), albumin (R = -0.31, p<0.01) and IgA (R = -0.32; p<0.01). No correlation was seen with age in the univariate analysis (R = 0.13; p = 0.29); or creatinine (R = 0.21; p = 0.01); IgM (R = 0.13; p = 0.11); AAG (R = -0.11; p = 0.20); or drugs with high protein binding (R = 0.01; p=0.93). The same correlations were observed in the individual different patient populations, except for the pediatric cohort, in which no significant correlation with IgA was observed (R = -0.22, p = 0.39).

Multiple linear mixed model analysis revealed that the strongest significant predictor for unbound vancomycin concentrations was the total concentration, completed by albumin in the pediatric cohort and albumin and serum IgA in the adult cohorts. The strongest significant predictor for unbound vancomycin concentration in the adult (i.e. hematology, ICU and orthopedic) and pediatric group was the total concentration (β = 0.626; standard error (SE) = 0.013; $P < 0.001$ and β = 0.806; SE = 0.032; P < 0.001; respectively). The R^2 of the final model for the adults and pediatric cohort were 0.952 and 0.866, respectively. Other variables found to be predictive for unbound vancomycin concentrations in the adult cohort included albumin (β = -0.162; SE = 0.019; $P < 0.001$) and serum IgA (β = -0.30; SE = 0.049; $P < 0.001$). On contrary, in the pediatric cohort, only albumin was found to be predictive for unbound vancomycin concentrations (β = -0.212; SE = 0.053; $P = 0.001$). No significant differences between the adult cohorts were found, with the same variable retained in the formula describing unbound vancomycin concentrations in the individual adult patient groups. The results of the multiple linear mixed model analysis for the separated groups are summarized in table 2. Based on these results, the unbound vancomycin concentration in adult and pediatric patients can be predicted with the following equations:

Adults: Unbound VAN (mg/L) = 0.63 x total VAN (mg/L) -0.30 IgA (g/L) -0.16 HA (g/L) + 5.57

Pediatric: Unbound VAN (mg/L) = 0.81 x total VAN (mg/L) - 0.21 HA (g/L) + 6.34

were total VAN conc represents the total vancomycin concentration in mg/L, IgA the immunoglobulin A concentration expressed in g/L and HA the human albumin concentration expressed in g/L.

Multiple linear mixed model analysis was also performed with bound vancomycin fraction (i.e. (total vancomycin concentration (mg/L) – unbound vancomycin concentration (mg/L))/ (total vancomycin concentration (mg/L))) as dependent variable. This analysis revealed the that the bound vancomycin concentration can be predicted by the albumin concentration ($\beta = 1.826$; SE = 0.271; $P < 0.001$) in the pediatric cohort and albumin ($\beta = 1.027$; SE = 0.076; $P < 0.001$) and IgA ($\beta = 1.717$; SE = 0.199; $P < 0.001$) in the adult cohort. Total vancomycin concentration was not retained as independent variable.

3.3. Model validation

Validation of the prediction model was evaluated in 13 adult patients. The median total vancomycin concentration was 15.4 mg/L (range: 11.6 to 31.8 mg/L). IgA and albumin concentrations ranged from 0.5 to 5.8 g/L and 18.8 to 38.8 g/L, respectively. The mean unbound vancomycin concentration was 8.8 mg/L (5.1 to 17.3 mg/L). The observed versus predicted plots for the patients included in the validation model are presented in figure 2. The R^2 was 0.99 (0.97-0.99), the mean difference was 0.2% (-14.2 - 13.8%) (figure 2).

4. Discussion

Our study adds interesting information on the current understanding of factors associated with unbound vancomycin concentrations. Compared to other studies, we examined relatively large patient populations and we evaluated additional factors that are potentially associated with unbound vancomycin concentrations and consequently vancomycin PPB, like IgA and total bilirubin concentrations or drugs with a PPB >70% in a large cohort of patients from different patient wards.[9,10,12,14] Moreover, we were able to measure unbound vancomycin concentrations by means of an LC-MS/MS method, validated for total as well as unbound concentrations, in contrast to other studies in which immunoassays (Fluorescence Polarization Immunoassay Techniques (FPIA) or Particle Enhanced Turbidimetric Inhibition Immunoassay (PETINIA)) or High Performance Liquid Chromatography (HPLC) with Ultra Violet (UV) detection were used.[9-14]

Although these methods offer sufficient sensitivity and selectivity for clinical purposes, they were developed and validated only for human plasma total drug concentrations. Also, it is well known that some immunoassay methods are prone to interference by paraproteins or rheumatic factor, [18] or can cross-react with crystalline degradation products, which comprise inactive metabolites of vancomycin found in plasma or serum samples from renally impaired and dialysis patients.[19-21] As LC-MS/MS methods are more specific, it is generally accepted that these methods are less likely to suffer from these issues.[22] Differences in unbound vancomycin concentrations between immunoassays and HPLC were recently illustrated in a study of Cradon *et al*, in which unbound vancomycin concentrations were measured by an FPIA and an in-house developed HPLC method.[9] Although the clinical significance of this difference remains debatable, they found a significantly lower and more variable PPB with the FPIA compared to HPLC.[9] Another study that evaluated PPB using HPLC and a PETINIA assay found a good correlation between both methods for measurement of unbound and total vancomycin concentrations. Of note, comparison with calculated PPB values were not reported.[11] Along with analytical issues (differences in matrix between total and unbound drug concentrations), also pre-analytical issues, like the conditions in which unbound vancomycin concentrations are obtained (temperature, pH, centrifugation speed and time) can have a major impact on unbound vancomycin

concentrations. A recent study described that the unbound vancomycin fraction after ultrafiltration at 4°C was on average 30.6% lower than after ultrafiltration at 37°C. Moreover, ultrafiltration at 37°C resulted in unbound vancomycin concentrations equivalent with equilibrium dialysis, which is considered as the gold standard for measuring unbound concentrations.[14] However, the latter method has the disadvantage that it needs several hours to reach equilibrium.[23] On the other hand, ultrafiltration is a fast and easy to perform method, but has the disadvantage of potential adsorption to the ultrafiltration filter. Here, we confirmed previous reports that non-specific adsorption of vancomycin to the ultrafiltration filter is negligible and independent of the concentration.

We found a median unbound vancomycin fraction of 61.7% (range: 45.9-95.9%) using UF at 37°C. These unbound vancomycin fractions are lower than those reported in the study by Stove *et al*, who reported values of 74.4% (SD: 6.6%).[14] These differences could possibly be attributable to the different methods that were used for measuring unbound and total vancomycin concentrations (immunoassay (FPIA) *vs* LC-MS/MS)). Secondly, vancomycin probably was measured in other patient populations, who could receive other drugs or endogenous molecules that could attribute to a different vancomycin protein binding.

The PPB characteristics of vancomycin have been studied in detail and found to be predominantly related to albumin and IgA content.[24,25] This is in line with our observations, as multiple linear mixed model analysis revealed a statistically significant correlation between unbound vancomycin concentrations and albumin and IgA in the total as well as separate adult datasets.

Our study clinically supports the results of other studies that have shown a negative correlation between serum IgA and unbound vancomycin concentrations.[14, 24-26] This is of particular interest in IgA myeloma patients, or in populations with low IgA concentrations (like selective IgA deficient patients, immunocompromised patients, etc.) as the high or low IgA concentrations will decrease or increase the unbound vancomycin concentration, and hence will reduce or increase the time above the MIC of unbound vancomycin, respectively.

We used a validated LC-MS/MS method for measuring total and unbound vancomycin concentrations. In our previous paper, we compared 4 different immunoassays with our LC-MS/MS method.[15] For some assays, acceptable agreement was obtained, making our formula theoretically valid for total

Antimicrobial Agents and Chemotherapy

vancomycin measurements with these assays. Commercially available immunoassays are however not validated for measuring unbound vancomycin concentrations in ultrafiltrate matrix. For instance, Cradon *et al* identified important differences in unbound vancomycin fractions between immunoassays and an in-house HPLC method.[9]

Previous studies on vancomycin PPB only included adult patients, whereas we were able to measure unbound vancomycin concentrations in a pediatric group. Although this group was rather small (11 patients), a significant difference in unbound vancomycin fractions was demonstrated compared to adult patients. This could in part be explained by the fact that pediatric patients have lower serum IgA concentrations.[28] Future studies in pediatric patients and neonates should confirm and further elucidate the difference in pediatric patient groups.

We found a significant and strong relationship between the total and unbound vancomycin concentration. This is similar to the results of other studies, which demonstrated that the unbound vancomycin concentration is highly predictable by the total concentration.[12,14] Compared to these studies, we evaluated additional potential covariates, like drugs with protein binding >70%. Although highly protein-bound drug are expected to be able to expel other drugs from albumin, thereby inducing elevated unbound concentrations, no significant correlation between vancomycin PPB and the amount of co-administered drugs with a PPB of more than 70% could be found in our study population.[28] Apart from a high PPB, drugs should have affinity for the same albumin binding places to expel vancomycin from the protein.[29] Frequently administered drugs in our study included NSAIDs (PPB >90%), aspirin (PPB > 99%), analgesics (PPB >95%) and vitamin K antagonists (PPB > 90%), drugs especially known for their ability to expel drug from albumin.[17,30] Future studies with vancomycin and albumin and known concentrations of the displacing agents should clarify if co-administration of highly protein binding drugs have an effect on unbound vancomycin concentrations.

In conclusion, we refined the current understanding of unbound vancomycin by measuring unbound and total vancomycin concentrations in different patient populations by means of a validated LC-MS/MS method. As we observed a significant correlation between total and unbound vancomycin concentrations in the four patients populations, measurement of unbound vancomycin concentrations seems to have no added value over measurements of total vancomycin concentrations. This implies

that dose changes based on unbound vancomycin concentrations would probably have been marginally different from dose changes based on routinely measured total vancomycin concentrations, except for patients with severe hypo- or hyperalbuminemia, IgA myeloma or IgA defiency, etc. We further developed and validated a formula based on IgA and albumin for adult patients to calculate the unbound vancomycin concentration. Although our formula was developed using LC-MS/MS, we expect the formula to be equally valid for immunoassays with a good agreement to our developed method for total vancomycin measurement. Last, we observed a significantly higher unbound fraction in the pediatric cohort. The clinical implications of this finding needs to be further examined.

Acknowledgements

We are grateful to Dr. Pharm K. Vanstraelen for the assistance with the statistical analysis of the data. Karel Allegaert, Pieter Vermeersch (fundamental clinical investigatorship 1800214 N and 1842013 N, respectively) and Steven Pauwels (Clinical fellowship 1700314N) are supported by the Fund for Scientific Research, Flanders.

5. References

1. **Finch RG, Eliopoulos GM. 2005.** Safety and efficacy of glycopeptide antibiotics. J Antimicrob Chemother **55:**Suppl 2: ii5-13.
2. **Liu C, Bayer A, Cosgrove SE, Daum RS, Fridkin SK, Gorwitz RJ, Kaplan SL, Karchmer AW, Levine DP, Murray BE, Rybak MJ, Talan DA, Chambers HF.** 2009. Clinical practice guidelines by the Infectious Diseases Society of America for the treatment of methicillin-resistant Staphylococcus aureus infections in adults and children. Clin Infect Dis **3**:e18-55.
3. **Steinkraus G, White R, Friedrich L.** 2007. Vancomycin MIC creep in non-vancomycin-intermediate Staphylococcus aureus (VISA), vancomycin-susceptible clinical methicillin-resistant S. aureus (MRSA) blood isolated from 2001-05. J Antimicrob Chemother **60**:788-794.
4. **Gould IM.** 2008. Clinical relevance of increasing glycopeptide MICs against Staphylococcus aureus. Int J Antimicrob Agents **31:**Suppl 2:1-9.
5. **Mouton JW, Theuretzbacher U, Craig WA, Tulkens PM, Derendorf H, Cars O.** 2008. Tissue concentrations: do we ever learn? J Antimicrob Chemother **61**:235-237.
6. **Lamer C, de Beco V, Soler P, Calvat S, Fagon JY, Dombret MC, Farinotti R, Chastre J, Gilbert C.** 1993. Analysis of vancomycin entry into pulmonary lining fluid by bronchalveolar lavage in critically ill patients. Antimicrob Agents Chemother **37**:281-286.
7. **Rybak MJ.** 2006. The pharmacokinetic and pharmacodynamic properties of vancomycin. Clin Infect Dis **42**:Suppl 1:s35-39.
8. **Bailey EM, Rybak MJ, Kaatz GW.** 1991. Comparative effect of protein binding on the killing activities of teicoplanin and vancomycin. Antimicrob Agents Chemother **35**:1089-1092.
9. **Cradon JL, Mac Vane SH, Nicolau DP.** 2013. Clinical laboratory-based assay methodologies may underestimate and increase variability of vancomycin protein binding in hospitalized patients. Pharmacotherapy **34:**203-209.
10. **Kees MG, Wicha SG, Seefeld A, Kees F, Kloft C.** 2014. Unbound fraction vancomycin in intensive care unit patients. J Clin Pharmacol **54**:318-323.

11. **Berthoin K, Ampe E, Tulkens PM, Carryn S.** 2009. Correlation between free and total vancomycin serum concentrations in patients treated for gram-positive infections. Int J Antimicrob Agents **34**:555-560.
12. **Butterfield JM, Patel N, Pai MP, Rosano TG, Drusano GL, Lodise TP.** 2011. Refining vancomycin protein binding estimated: identification of clinical factors that influence protein binding. Antimicrob Agents Chemother **55**:4277-4282.
13. **Ampe E, Delaere B, Hecq JD, Tulkens PM, Glupczynski Y.** 2013 Implementation of a protocol for administration of vancomycin by continuous infusion: pharmacokinetic, pharmacodynamics and toxicological aspects. Int J Antimicrob Agents **41**:439-446.
14. **Stove V, Coene L, Carlier M, De Waele JJ, Fiers T, Verstraete AG.** 2015. Measuring unbound versus total vancomycin concentrations in serum and plasma: methodological issues and relevance. Ther Drug Monit **37**:180-187.
15. **Oyaert M, Peersman N, Kieffer D, Deiteren K, Smits A, Allegaert K, Spriet I, Van Eldere J, Verhaegen J, Vermeersch P, Pauwels S.** 2015. Novel LC-MS/MS method for plasma vancomycin: comparison with immunoassays and clinical impact. Clin Chim Acta **441**:63-70.
16. European Medicines agency, guideline on bioanalytical method validation. 21 July 2011.
17. **Hardman JG, Limbird LE, Goodman Gilman A (ed).** Goodman and Gilman's the pharmacological basis of therapeutics, 10th ed., p 1924-2023. R.R. Donnelley and Sons, Chicago IL.
18. **LeGatt DF, Blakney GB, Higgins TN, Schnabl KL, Shalapay CE, Dias VC, Wesenberg JC.** 2012. The effect of paraproteins and rheumatoid factor on four commercial immunoassays for vancomycin: implications for laboratorians and other health care professionals. Ther Drug Monitor **34**:306-311.
19. **Anne L, Hu M, Chan K, Colin L, Gottwald K.** 1989. Potential problem with fluorescence polarization immunoassay cross-reactivity to vancomycin degradation product CDP-1: its detection in sera of renally impaired patients. Ther Drug Monit **11**:585-591.
20. **Robert WL.** 1999. Crystalline degradation product cross-reactivity with vancomycin fluorescence polarization immunoassay. *Pharmacotherapy* **19**:1467-1468.

21. **Somerville AL, Wright DH, Rotschafer JC.** 1999. Implications of vancomycin degradation products on therapeutic drug monitoring in patients with end stage renal disease. Pharmacotherapy **19**:702-707.

22. **König K, Kobold U, Fink G, Leinenback A, Dülffer T, Thiele R, Zander J, Vogeser M.** 2014. Quantification of vancomycin in human serum by LC-MS/MS. Clin Chem Lab Med **51**:1761-1769.

23. **Zeitlinger MA, Derendorf H, Mouton JW, Cars O, Craig WA, Andes D, Theuretzbacher U.** 2011. Protein binding: do we ever learn? Antimicrob Agents Chemother **55**:3067-3074.

24. **Sun H, Maderazo EG, Krusell AR.** 1993. Serum protein-binding characteristics of vancomycin. Antimicrob Agents Chemother **37**:1132-1136.

25. **Cantu TG, Dick JD, Elliott DE, Humphrey RL, Kornhauser DM.** 1990. Protein binding of vancomycin in a patient with immunoglobulin A myeloma. Antimicrob Agents Chemother **34**:1459-1461.

26. **Ackerman BH, Taylor EH, Olsen KM, Abdel-Malak W, Pappas AA.** 1988. Vancomycin serum protein binding determination by ultrafiltration. Drug Intell Clin Pharm **22**:300-303.

27. **Stoop JW, Zegers BJM, Sander PC, Ballieux RE.** 1969. Serum immunoglobulin levels in healthy children and adults. Clin Exp Immunol **4**:101-112.

28. **Johnson GJ, Kilpatrick CJ, Bury RW, Fullinfaw RO, Moulds RF.** 1989. Unbound phenytoin plasma concentrations in patients co-medicated with sodium valproate-the predictive value of plasma albumin concentration. Br J Clin Pharmacol **27**:843-849.

29. **Koch-Weser J, Sellers EM.** 1976. Drug Therapy. Binding of drugs to serum albumin (second of two parts). N Engl J Med **294**:526-31.

30. **Vanstraelen K, Wauters J, Vercammen I, de Loor H, Maertens J, Lagrou K, Annaert P, Spriet I.** 2014. Impact of hypoalbuminemia on voriconazole pharmacokinetics in critically ill adult patients. Antimicrob Agents Chemother **58**:6782-6789.

460 **Table 1.** Demographics, clinical and biochemical characteristics at day of sampling of patients treated with vancomycin. Data are presented as median and
461 range.

	Intensive care	Hematology	Orthopedic	Pediatrics	Total
Number of patients	33	22	24	11	90
Age (years) (range)	59 (17-80)	63 (17-81)	66 (32-84)	3 (1-14)	61 (1-84)
Male / Female (number)	19/16	9/13	15/9	7/4	50/40
Number of samples	51	33	44	18	146
albumin (g/L)	28.4 (16.7-39.2)	29.4 (18.2-37.5)	35.0 (21.0-46.5)	28.9 (20.0-39.5)	31.4 (16.7-46.5)
IgA (g/L)	3.5 (0.2-6.8)	1.2 (0.7-12.3)	1.9 (0.4-7.15)	0.4 (0.01-1.0)	1.7 (0.0-12.3)
IgM (g/L)	0.8 (0.1-2.8)	0.3 (0.1-5.4)	0.5 (0.3-1.7)	0.5 (0.1-1.2)	0.5 (0.1-5.4)
AAG (g/L)	1.8 (0.4-3.9)	2.1 (0.8-3.0)	1.4 (0.4-2.5)	1.8 (1.0-3.9)	1.7 (0.4-3.9)
Total protein (g/L)	55.0 (38.0-90.0)	60.5 (45.0-83.0)	61.2 (49-89)	52.0 (46.0-67.0)	58.4 (38.0-90.0)
Total bilirubin (mg/dL)	0.68 (0.1-35.9)	0.24 (0.01-3.69)	0.12 (0.008-1.9)	0.19 (0.11-0.90)	0.2 (0.0-35.6)
urea (mg/dL)	80.0 (7.0-218))	27.0 (8.0-85.0)	34.0 (12-140)	9.5 (3.0-93.0)	33.0 (3.0-218.0)
creatinine (mg/dL)	1.3 (0.1-10.0)	0.6 (0.2-3.3)	1.0 (0.6-1.9)	0.2 (0.1-0.9)	0.8 (0.1-10.0)
eGFR (ml/min/1.73m^2) – CDK-EPI	64.9 (6.0 - >90)	>90 (14.1- >90)	79 (22.6- >90)	>90	>90 (6.4->90)
Total vancomycin concentration (mg/L)	17.8 (3.6-53.2)	11.8 (4.5-24.4)	13.0 (6.2-27.8)	10.7 (0.7-25.1)	13.5 (0.7-53.2)
Unbound vancomycin concentration (mg/L)	10.9 (2.3-34.2)	7.7 (2.2-20.8)	7.4 (3.2-16.1)	8.2 (.6-22.6)	8.5 (0.6-34.2)
Unbound vancomycin fraction (%)	61.7 (47.0-87.6)	60.6 (48.7-90.6)	56.4 (45.9-78.0)	81.3 (61.9-95.9)	61.7 (45.9-95.9)
Number of co-administered drugs with PPB >70%	7.0 (1.0-16.0)	8.0 (1-14)	6.0 (2.0-16.0)	4.0 (0.0-12.0)	6.0 (0.0-16.0)

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463 **Table 2.** Overview of the prediction model parameters estimating unbound vancomycin
464 concentrations in the different patient populations. β: beta-coefficient; SE: Standard error
465

Variable	β[a]	SE[b]	*P*
Hematology			
Total vancomycin (mg/L)	0.643	0.037	< 0.001
Albumin (g/L)	-0.222	0.035	< 0.001
Serum IgA (g/L)	-0.130	0.059	< 0.001
Coefficient	6.757		
Intensive Care			
Total vancomycin (mg/L)	0.637	0.020	< 0.001
Albumin (g/L)	-0.179	0.048	0.001
Serum IgA (g/L)	-0.334	0.113	< 0.001
Coefficient	6.269		
Orthopedic			
Total vancomycin (mg/L)	0.657	0.030	< 0.001
Albumin (g/L)	-0.117	0.029	< 0.001
Serum IgA (g/L)	-0.252	0.105	0.009
Coefficient	4.950		
Total adult population			
Total vancomycin (mg/L)	0.626	0.013	<0.001
Albumin (g/L)	-0.162	0.019	<0.001
Serum IgA (g/L)	-0.300	0.049	<0.001
Coefficient	5.573		
Pediatrics			
Total vancomycin (mg/L)	0.806	0.032	< 0.001
Albumin (g/L)	-0.212	0.053	0.001
Coefficient	6.340		

Figure 1. Box and whisker plot showing the distribution of unbound vancomycin fractions (**A**) and total vancomycin concentrations (**B**) for the different patient populations.

The data are reported as boxes indicating the 10^{th}, 25^{th}, 50^{th} (median), 75^{th} and 90^{th} percentiles of vancomycin. The levels of statistical significance ($p < 0.05$, Mann Whitney U Test) are indicated in the figure. Statistically non-significant differences are not indicated in the figure.

Figure 2. Validation of our prediction model. **Panel A.** Passing Bablok regression analysis of the observed unbound vancomycin concentration and predicted unbound vancomycin concentration in 13 adult patients. **Panel B.** Bland-Altman analysis of measured unbound vancomycin concentration plotted against predicted unbound vancomycin concentration. Horizontal dashed lines are drawn at the mean difference (mg/L) and at the limits of agreement.